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BY

LIEUT.-COLONEL W. B. BANNERMAN, M.D., B.Sc., I.M.S.

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THE PRODUCTION OF ALKALI IN LIQUID MEDIA BY THE BACILLUS PESTIS.

THE fact that plague microbes cease to grow abundantly in liquid media after a month or six weeks has hitherto been ascribed to the supposed consumption of all the available nutriment. Thus, Mr. Haffkine*, in his evidence before the Indian Plague Commission in 1898, says the broth becomes modified in the course of five or six weeks, so that "the liquid is exhausted, its nutritious elements having been incorporated in the body of the bacilli" ....." It will not feed any further growth."

Dr. E. Klein† also assumes that the growth of plague bacilli stops because the nutritive material in the broth is exhausted.

In this laboratory it has been common experience to find that not only does active growth cease in flasks containing 1000 c.c. or even 2000 c.c. of broth after six weeks or two months, but that the plague germs actually die out altogether in from two to four months' time.

As Mr. Haffkine taught that an acid medium is inimical to the growth of the plague germ, a trial was made to see if the medium had become acid. To our surprise we found that, on the contrary, the medium in these old sterile flasks had become highly alkaline, many of them containing the equivalent of from 2 to 2·5 per cent. of normal alkali.

Macé says on this point with regard to the plague bacillus (p. 925) "Le microbe produit un peu d'acide dans les milieux, probablement aux dépens des hydrocarbones."‡

That this alkali was not due to any change taking place in the broth itself was proved by testing a flask containing sterile broth made seven months previously. The reaction given by this broth was the amphoteric reaction to litmus paper which is given by the freshly made media used here.

To prove whether the alkaline condition of the broth was the cause of the cessation of the growth the following experiment was made.

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* Minutes of evidences taken by the Indian Plague Commission with appendices, Vol. I, pp. 2, 4, 28. Calcutta; Office of the Superintendent of Government Printing, India, 1900.

† Thirty-first Annual Report of the Local Government Board, 1901-1902. Supplement containing the Report of the Medical Officer for 1901-1902.

‡ Traité Pratique de Bactériologie par E. Macé, Cinquiéme édition, Paris: Libraire J. B. Ballière et Fils, 1904.

Six flasks containing 1000 c.c. of broth, which had been sown with plague four months previously, were tested for plague growth and reaction with the following result:

Growth on agar.	No. of c.c. of normal acid per litre required to produce neutralisation.
I. Sterile	14·7
II. „	22·2
III. „	25·2
IV. „	24·5
V. „	23·5
VI. „	24·1

That is to say, in four months' growth the plague germ had produced alkalinity in the broth equivalent to from $1\frac{1}{5}$ to $2\frac{1}{5}$ per cent. of normal alkali as tested by the following procedure:

Ten c.c. of the neutral broth used in the laboratory, which was amphoteric to blue and red litmus papers, was diluted with 90 c.c. of distilled water in a porcelain dish. To this was added 2 c.c. of solution of litmus made according to the directions in the British Pharmacopœia.

The tint thus produced was taken as the measure of the neutrality with which to compare the fluid from the old plague flasks.

Ten c.c. from each of the above-mentioned flasks was withdrawn by a sterile pipette, and added to 90 c.c. of distilled water; 2 c.c. of litmus solution was added, and $\frac{N}{20}$ acid added from a burette till the tint corresponded to that of the standard broth. As a slight precipitate was produced when the acid was added to the plague broth, this first dish of tested broth was kept as the standard tint to be aimed at as being easier to match than the pure neutral broth.

It is allowed that the above method of arriving at the neutral point of the broth or plague culture is not ideal, but at the time (1904) no more perfect method had been found for the broth then used in the laboratory.

Phenolphthalein and methyl orange were both tried but proved less satisfactory than the litmus.

These six flasks were, on 27th June 1904, resown with plague germs after having been neutralised with hydrochloric acid.

Agar cultures made from them, on 4th July 1904, *i.e.*, seven days later, showed a good growth of plague in all, with the exception of No. III, which had become contaminated.

When again tested on the 11th July the following were the results—

I	was strongly alkaline to litmus paper,	and showed	good growth	of plague on agar.
II	Ditto	ditto	ditto	ditto.
IV	was slightly alkaline to litmus paper,	and showed	good growth	of plague on agar.
V	Ditto	ditto	ditto	ditto.
VI	Ditto	ditto	ditto	ditto.

These flasks were again tested on 22nd July 1904, *i.e.*, three weeks from the date of re-sowing, with the following results:

I	contained	1·2	per cent.	alkali	and produced	good growth of plague on agar.
II	„	1·17	„	„	ditto	ditto.
IV	„	1·15	„	„	but proved to be contaminated.	
V	„	1·0	„	„	and produced	good growth of plague on agar.
VI	„	1·16	„	„	ditto	ditto.

The plague germs in the re-sown flasks had in three weeks produced as much alkali as though growing in fresh nutrient medium.

This conclusively proves that the broth is not "exhausted" but still contains nutriment sufficient for the nourishment of a new generation of plague germs.

Another proof that the growth of plague is stopped by the alkalinity of the medium is afforded by the following experiment.

A series of flasks containing 100 c.c. of broth was prepared by adding 0·25, 0·5, and 0·75 per cent. of normal hydrochloric acid to the amphoteric medium as used in the laboratory, and another set by similarly adding 0·25, 0·5, 0·75, 1·0, 1·5, and 2 per cent. of normal sodium hydroxide.

These flasks and also two control flasks of amphoteric broth as ordinarily used in the laboratory were sown with 0·5 c.c. of a 3 days' old culture of plague on 24th June 1904.

The subsequent history of these flasks is shown below.

25th June 1904.—The amphoteric broth flasks showed dots of growth on the sides of the glass, and commencing stalactite formation.

The 0·25	acid broth	showed	dots	but no stalactites.
The 0·50	„	„	„	a few dots only.
The 0·75	„	„	„	no growth at all.
The 0·25	alkaline broth	showed	dots	but no stalactites.
The 0·50	„	„	„	a few dots only.
The 0·75	„	„	„	a fewer number of dots.

Those of the higher series showed no growth at all.

26th June 1904.—The amphoteric broth flasks showed good stalactite formation.

The 0·25	acid broth	showed	growth as good as the above.	
The 0·50	„	„	„	abundant dots and a few stalactites.
The 0·75	„	„	„	„ „ only.

The 0·25 alkaline broth showed abundant dots and a few stalactites.

The 0·50, The 0·75, The 1·00 } show similar growth but in a less degree, and the growth is distinctly less in the broths of higher alkalinities.

The flasks of 1·5 and 2 per cent. acid broth show no growth at all.

27th June 1904.—The amphoteric broth shows abundant stalactite growth.

The 0·25 acid broth shows equally good growth.
The 0·50 „ „ „ excellent growth of stalactites.
The 0·75 „ „ „ one stalactite and numerous dots.
The 0·25 alkaline broth shows good growth of stalactites.
The 0·50 „ „ „ „ „ „ „
The 0·75 „ „ „ slightly less growth.
The 1·00 „ „ „ still less growth.
The 1·50 „ „ „ about a dozen dots only.
The 2·00 „ „ „ no growth at all.

Another similar experiment was made with flasks containing 100 c.c. of broth acidulated by the addition of 0·5, 0·75, 1·0, 1·25, 1·5, and 2 per cent. of normal acid.

A corresponding set of flasks was treated by the addition of similar quantities of normal alkali.

On 8th July 1904 to each flask was added 0·1 c.c. of plague culture in broth, and along with control flasks all were put aside to observe the growth. Three days later the flask with amphoteric broth and that with the 0·5 per cent. of acid showed a growth of abundant long stalactites, and it was difficult to say which was best.

The flask with the 0·75 per cent. acid broth showed short stalactites and many dots of growth on the glass.

This latter appearance was also seen on the sides of the other acid flasks, but no stalactites were visible at this stage. The flasks with alkaline broth showed much inferior growths. Thus, the 0·75 alkaline broth showed one or two short stalactites and dots on the glass, but nothing like the corresponding acid flask. The higher series of alkaline broths showed dotting on the glass merely and no stalactites.

From the above it is clear that, far from being inimical to the growth of the plague germ, slightly acid broth is a favourable medium for its growth. It is likewise clear that an alkaline medium is less favourable to its growth, and that a broth containing the equivalent of 2 per cent. of normal alkali inhibits the growth altogether. It does not, however, kill the plague germ, for if a few drops from such a flask are sown on agar a growth of plague is obtained, and from old

prophylactic flasks that have stood in the incubation room for eighteen months, and have a reaction approaching 2 per cent. of normal alkali, germs can still be recovered sufficiently alive to show growth on agar slopes.

A question naturally arising from the above is : can the plague bacillus evolve alkali when grown in acid broth so as to produce a neutral and finally an alkaline broth ?

To solve this the following proceeding was adopted. The first set of the above series of flasks was shaken up on 9th July 1904. When examined on 11th July 1904, it was found that the best stalactites were to be seen in the flask containing broth originally acidulated to the extent of 0·75 per cent. On testing the fluid in this flask it was found that it now contained the equivalent of 0·15 per cent. of normal alkali, therefore the plague bacilli had made enough alkali in the eighteen days they had been growing to neutralise all the acid introduced, and had produced in addition 0·15 per cent. of normal alkali and this would no doubt have gone on until the 2 per cent. limit of alkalinity was reached.

Similarly, the plague germs growing in the flask containing the 0·5 per cent. acid broth were found to have produced 1·05 per cent. ; and the germs growing in the 0·25 per cent. broth, 0·6 per cent. of alkali.

Two flasks containing a similar quantity of amphoteric broth and sown with the same germ had in the same eighteen days produced the equivalent of 0·40 and 0·35 per cent. of alkali respectively.

In the series of alkaline broth flasks the following results were obtained.

Flask with 0·75 per cent. of normal alkali added, showed a deficiency in alkali to the extent of 0·01 per cent. Flask with 1·0 per cent. of normal alkali added, showed a deficiency in alkali to the extent of 0·35 per cent. Flask with 1·5 per cent. of normal alkali added, showed a deficiency in alkali to the extent of 0·75 per cent. Flask with 2 per cent. of normal alkali added, showed a deficiency in alkali to the extent of 0·91 per cent.

These two latter flasks though showing no stalactites but only a muddiness after the eighteen days' incubation, yet gave a good growth of plague when a few drops were placed on agar, proving that the germs were still alive though their growth had been inhibited.

A second observation in confirmation of the above may be recorded. The series of flasks noted above as sown on 8th July 1904 were tested on 29th July 1904. The growth was undoubtedly best developed in the flask containing 1 per cent. acid broth. There was little growth in the 1·25 per cent. acid broth and none apparently in the higher numbers of the acid series. A precipitate was, however, evident in these highly acidulated broths as well as in others of the same degree of acidulation which had not been sown with plague.

Cultures taken from this whole series showed a pure growth of plague in all

the alkaline broths, and in the less acid ones up to 1·0 per cent.; the 1·25 and 2·0 per cent. acid broths showed no growth at all on agar.

The results got by titration of this series, as well as those of the first experiment, are shown below in percentages.

Denomination of flask.	Result of titration.	Alkali produced.	Alkali produced in first experiment.
2 per cent. acid . .	2·0 per cent. acid.	0	
1·50 „ „ „ . .	1·38 „ „ „	0·12	
1·25 „ „ „ . .	1·26 „ „ „	0	
1·00 „ „ „ . .	0·26 „ „ ,	0·74	
0·75 „ „ „ . .	0·48 „ „ alkali.	1·23	0·90
0·50 „ „ „ . .	0·67 „ „ „	1·17	1·05
Amphoteric . . .	0·49 „ „ „	0·49	0·42 0·35
0·75 per cent. alkali .	1·04 „ „ „	0·29	—0·01
1·00 „ „ „ . .	1·13 „ „ „	0·13	—0·35
1·25 „ „ „ . .	1·31 „ „ „	0·06	—
1·50 „ „ „ . .	1·41 „ „ „	—0·09	—0·75
2·00 „ „ „ . .	1·35 „ „ „	—0·65	—0·91

One flask containing 2 per cent. acid broth which had not been sown was tested nine days after preparation and found on titration by the method described above, to give an acidity of 1·89 per cent. only. Three flasks containing broth with alkalinities of 1·0, 1·5, and 2 per cent., respectively, were tested after nine days and found to show a loss of 0·07, 0·26, and 0·39 of alkali respectively.

A series of flasks containing 1000 c.c. of broth was tested to see at what rate alkali was produced. The following shows the results for weekly periods after sowing of the plague germ.

Number of weeks since sowing.	Percentage of normal alkali produced since sowing.
1	0·41
2	0·55
3	1·11
4	1·38
5	1·42
6	2·10

The conclusion, then, appears to be that—

1. In the higher series of both acid and alkali broths, the production of alkali by the plague germ is stopped.
2. In the higher alkaline series the plague germ lives for a long time, whereas in the highly acid broths it quickly dies out.
3. The more alkaline the medium is, the less alkali is produced by the plague germs sown in it, and in the higher series little or none is produced, the growth being apparently inhibited.
4. The production of alkali is regular, increasing from week to week to the maximum, which is attained in about six weeks.

The above investigation was carried out in 1904, but owing to the labour involved in organising and working this laboratory, and the urgency of other work, nothing further could be done till this year. In the meantime the technique used in making the broth had been changed, and it was found possible to use the more accurate method of titration with phenolphthalein as recommended by the Committee of American Bacteriologists. The broth used in the laboratory is prepared by chemical digestion with hydrochloric acid, no artificial peptone being added, so that no offence might be occasioned to the religious prejudices of certain sections of the Indian community. The details were worked out by the late Colonel Warden, I.M.S., and involved digestion under steam pressure for six hours. The medium so prepared had a very high colour and varied much in composition. Various alterations were made from time to time, such as reduction in the amount of acid, shortening the time of, and lessening the pressure during, digestion. It was found, however, that heating under pressure led to the production of other bodies than albumose and peptone, as proved by heating a sample of the broth with caustic alkali, when a strong smell of ammonia and methylamine bodies was evolved.

These considerations led to the trial of a new method of preparation where length of maceration at a low temperature was substituted for heating for a short time at a high temperature under pressure. The experiments on which these opinions regarding composition of the broth are founded were devised and carried out by Mr. F. Maitland Gibson, M.B., B.Sc., of this laboratory, to whom is due the whole credit in the matter.

The conclusion arrived at was that broth prepared according to the following method was better for the purpose of growing the plague bacilli than any used hitherto, that it was equal in nutritive value to bouillon made in the ordinary way to which artificial peptone had been added, and that it no longer gave off ammonia when heated with caustic alkali.

To each kilogramme of minced and fat-free goat's flesh, 80 c.c. of hydro-

chloric acid of the British Pharmacopœia is added, and the whole is digested in a stoneware jar in a water bath at 70° Cent. for three days.

The mixture of hydrolysed meat and acid is then mixed with water in the proportion of two litres of water to one of mixture, the whole rendered neutral to litmus paper with sodium hydroxide, and then boiled for one hour at atmospheric pressure.

The mixture is then strained through cloth, the reaction adjusted so as to be slightly alkaline to litmus paper, and filtered into 4-litre flasks. One litre of fluid only is placed in each flask, so that a large surface is exposed to the air. Two drops of fresh cocoanut oil are added and the whole sterilised for twenty minutes at one atmosphere of pressure.

With broth prepared as above, it was found possible to use phenolphthalein as an indicator. The medium as used in the laboratory is adjusted to an arbitrary standard by means of litmus paper, and on testing it with phenolphthalein it is found to be acid to the extent of 1·5 per cent. A series of broths of different brews was tested and the results obtained varied only very slightly from this figure. It may be taken, therefore, that all the fluid medium used has this reaction when sent up from the kitchen.

The procedure was as follows. Five c.c. of medium was placed in a six-inch porcelain evaporating basin, to this was added 45 c.c. of distilled water and 1 c.c. of a 0·5 per cent. solution of phenolphthalein in 50 per cent. alcohol. The whole was heated to boiling and then titrated as rapidly as possible with a $\frac{N}{20}$ solution of sodium hydroxide. The first change in colour produced by this addition must not be mistaken for the proper pink required. The first colour produced is a dull tint called "Italian Pink" by the American Committee,* but on addition of more alkali a clear bright pink colour is suddenly developed. This was regarded as the end point of titration and was always estimated *immediately after boiling* before the fluid had cooled, as it is found that the pink colour becomes more intense as cooling takes place.

Working in this way a series of observations was made to see whether the plague germ would produce the same amount of alkali when grown in the broth made in the new manner, as we have seen that it did in the broth as originally prepared.

A sample of the new broth having been sown with plague germs, was tested from week to week with the following results:

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* Procedures recommended for the study of Bacteria, etc., being a Report of a Committee of American Bacteriologists to the Committee of the Pollution of Water Supplies of the American Public Health Association. Concord, N. H., the Runford Press, 1898.

Time.	Per cent. normal acid present.	Per cent. normal alkali produced.
At start	=1·6 per cent. normal acid	0·0
After 9 days	=1·2 „ „ „ „	·4
„ 19 „	=1.15 „ „ „ „	·45
„ 31 „	=1·1 „ „ „ „	·5
„ 46 „	=0·0 „ „ „ „	1·6

To render a comparison possible between the old method of titration and the new, the following procedure was adopted.

Unsown broth and a four-month-old broth plague culture were tested with litmus and phenolphthalein, with the result set out in the following table:

Substance tested.	Reaction to litmus.	Reaction to phenolphthalein.
Unsown broth . .	0·79 per cent. normal alkali .	2·41 per cent. normal acid.
Four-month-old broth plague culture . .	2·68 per cent. normal alkali .	0·69 per cent. normal acid.
Alkali produced . .	1·89 per cent. normal alkali	1·72 per cent. normal alkali.

It would appear, then, that the plague germ produces alkali in the broth as at present used, just as it did in the broth as formerly prepared and to very much the same extent.

To test once more the theory that it is the presence of alkali that causes cessation of growth of the plague germ the following experiment was carried out.

Twelve flasks of anti-plague vaccine in process of incubation were selected from the stock at that time in the laboratory. The germs with which these flasks had been sown were of different strains from the following sources. Germs 2 and 3 were from the heart blood of a rat, 4 and 5 from the heart blood of a guinea-pig, and 6 from the heart blood of a monkey.

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The reaction of these flasks was tested on 11th July 1907 with the following results:

Flask No.	Germ sown.	Date of sowing.	Period of incubation.	Reaction of phenolphthalein per cent normal acid.	Percentage normal alkali produced taking start point = 1·5 % normal acid.
1	2	15th April 1907	87 days	0·18	1·32
2	2	„	87 „	0·12	1·38
3	2	16th April 1907	86 „	0·28	1·22
4	3	23rd April 1907	79 „	1·21	0·29
5	3	„	79 „	1·60	...
6	4	14th May 1907	58 „	0·67	0·83
7	4	20th May 1907	52 „	0·34	1·16
8	5	25th May 1907	47 „	0·89	0·61
9	5	„	47 „	0·57	0·93
10	5	„	47 „	0·77	0·73
11	6	30th May 1907	42 „	0·66	0·84
12	6	„	42 „	0·48	1·02

The flasks were neutralised (with the exception of 4 and 5) by the addition of the calculated quantity of normal HCl.

The flasks were tested from week to week, and it was found that the amount of alkali again gradually increased as in the former experiment.

The final result after thirty-seven days is given below.

Flask No.	Reaction to phenolphthalein per cent. normal acid.	Alkali produced.
1	0·75	0·75
2	0·60	0·90
3	0·75	0·75
4	0·55	0·66 *
5	1·00	0·60 *
6	0·35	1·15
7	0·45	1·05
8	0·41	1·09
9	0·47	1·03
10	0·50	1·00
11	Contaminated	—
12	0·67	0·83

* These flasks had not been neutralised (as noted above) as they were found to differ very slightly from the broth medium when first tested along with the others. The alkali produced is calculated from the reaction at the first test.

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The amount of alkali produced as stated in column 3 is calculated from he start point which should have been obtained by the addition of the calculated amount of normal acid, *i.e.*, equal to 1·5 per cent. normal acid.

But it was discovered that the quantity of acid added was not quite sufficient actually to produce this result, so that the amount of alkali obtained as stated opposite each flask in the above table is slightly more than the germ really formed (*cf.* para. 2, p. 6, *supra.*)

The idea that a broth treated as above, *i.e.*, neutralised after the formation of alkali so as to allow the plague germ to have a fresh term of active growth, would be more efficient as a prophylactic, was suggested. A trial of a vaccine made from such twice grown broth was made in 1904, but no advantage was apparent as regards prophylactic property when contrasted with the ordinary prophylactic made at the same time.

What is this alkali? Is it a metabolic product of the plague germ, and if so, has it anything to do with the toxin? That the twice grown broth was no more efficacious than the ordinary vaccine might be due to the acid added having destroyed its immunising property—supposing it to have any. But as the fluid part of the vaccine appears to have only slight power of conferring immunity, it is probable that the alkaline material is not connected with the substance producing immunity.

SUMMARY.

1. The plague germ produces alkali when grown in the ordinary broth media as made in the Bombay Bacteriological Laboratory.

2. This alkaline condition of the broth causes the stoppage of growth of the plague germ. The ultimate alkalinity reached is equivalent to about 1·5 to 2·5 per cent. of normal sodium hydroxide.

3. The plague germ, however, may remain alive for at least eighteen months in such media, its growth being inhibited merely, death not occurring.

4. If the broth which has been rendered alkaline by the growth of the plague germ is neutralised, a fresh growth takes place and continues till the alkalinity reaches the equivalent of 1·5 to 2·5 per cent. of normal sodium hydroxide.

5. The more alkaline the broth medium is made, the less alkali is produced by plague germs sown in it, and, in a highly alkaline broth, growth ceases altogether, though the germs may remain alive.

6. The alkali increases regularly from week to week, reaching a maximum in six weeks or two months.

7. Broth slightly acid to phenolphthalein is more favourable to the growth of the plague germ than an alkaline one.

8. A highly acid broth is inimical to the growth of the plague germ, and it soon dies out in such a medium.

9. The alkaline body is probably not the plague toxin.

In conclusion, I desire to acknowledge the unstinting and generous help afforded in the first part of this research by my friend and colleague, Captain W. Glen Liston, M.D., I.M.S., and in the latter by my friend, Mr. A. R. Normand, B.Sc., Professor of Chemistry in the Wilson College, Bombay, without whose expert chemical knowledge the work would have been well nigh impossible.

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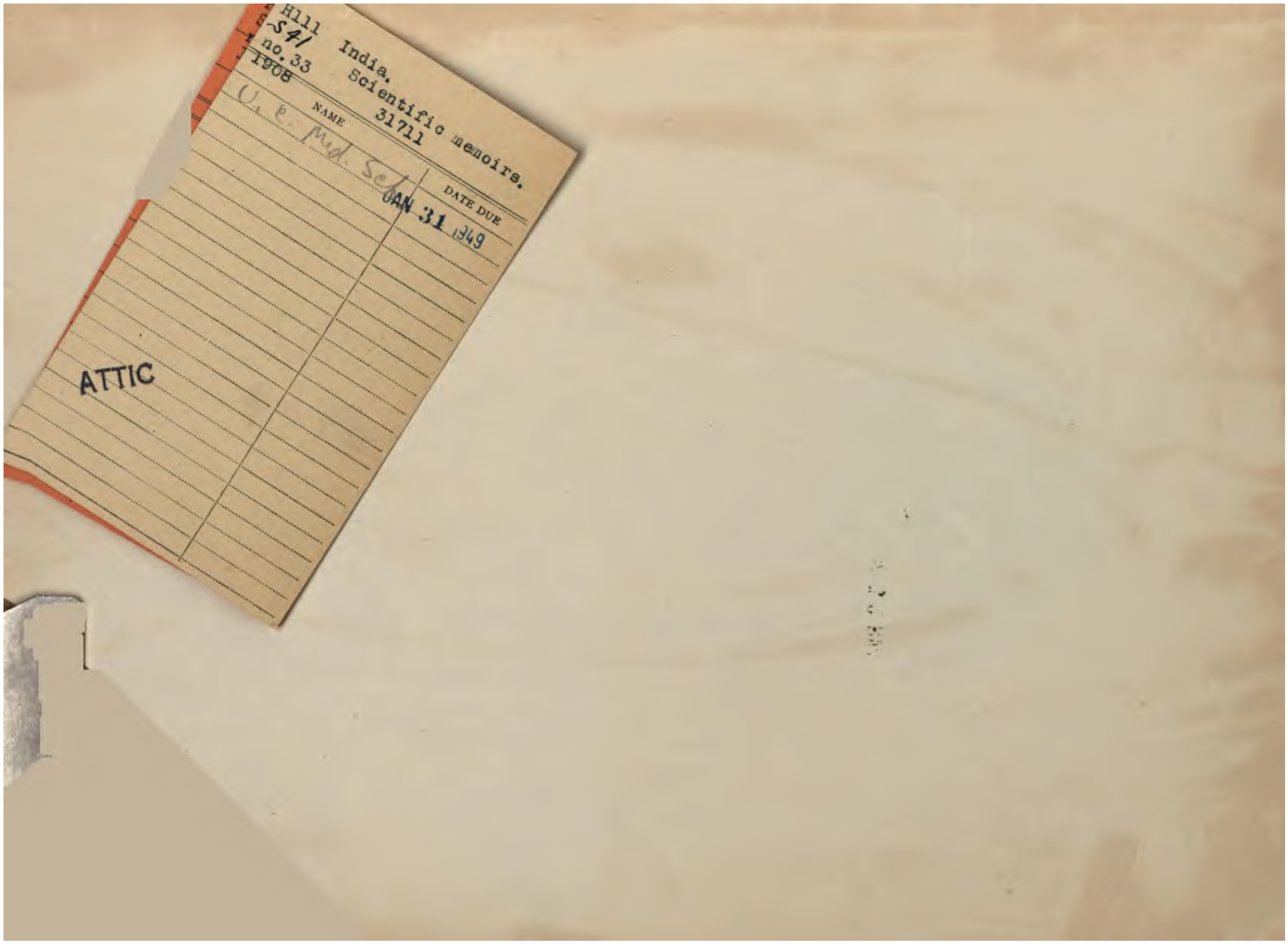
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